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Anaerobic Degradation of Toluene and *o*-Xylene by a Methanogenic Consortium†

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Toluene and *o*-xylene were completely mineralized to stoichiometric amounts of carbon dioxide, methane, and biomass by aquifer-derived microorganisms under strictly anaerobic conditions. The source of the inoculum was creosote-contaminated sediment from Pensacola, Fla. The adaptation periods before the onset of degradation were long (100 to 120 days for toluene degradation and 200 to 255 days for *o*-xylene). Successive transfers of the toluene- and *o*-xylene-degrading cultures remained active. Cell density in the cultures progressively increased over 2 to 3 years to stabilize at approximately 10^9 cells per ml. Degradation of toluene and *o*-xylene in stable mixed methanogenic cultures followed Monod kinetics, with inhibition noted at substrate concentrations above about 700 μM for *o*-xylene and 1,800 μM for toluene. The cultures degraded toluene or *o*-xylene but did not degrade *m*-xylene, *p*-xylene, benzene, ethylbenzene, or naphthalene. The degradative activity was retained after pasteurization or after starvation for 1 year. Degradation of toluene and *o*-xylene was inhibited by the alternate electron acceptors oxygen, nitrate, and sulfate. Degradation was also inhibited by the addition of preferred substrates such as acetate, H_2, propionate, methanol, acetone, glucose, amino acids, fatty acids, peptone, and yeast extract. These data suggest that the presence of natural organic substrates or cocontaminants may inhibit anaerobic degradation of pollutants such as toluene and *o*-xylene at contaminated sites.

Soil, sediment, and groundwater are frequently contaminated with petroleum products as a result of leaks in underground storage tanks, improper disposal techniques, and inadvertent spills. Of the many constituents of petroleum, the nonoxygenated, homocyclic aromatic compounds (including benzene, toluene, xylenes, and ethylbenzene) are of particular concern because they are confirmed or suspected carcinogens, even at very low concentrations (6). These compounds are relatively water soluble compared with other components of petroleum and thus frequently migrate through groundwater systems to contaminate drinking water supplies far removed from the actual spill (5, 29). The microbial degradation of compounds such as toluene and benzene under aerobic conditions has been studied in great detail (13, 30); in contrast, the fate of homocyclic aromatic compounds in anaerobic environments is very poorly understood. Until the mid-1980s, it was generally believed that monoaromatic compounds were recalcitrant to degradation under anaerobic conditions. Certain monoaromatic hydrocarbons, most frequently toluene, have since been shown to be degraded by microorganisms under denitrifying (8, 11, 20, 21, 25, 36), iron-reducing (22, 23), sulfate-reducing (2, 10, 17) and methanogenic (16, 32, 33, 34) conditions. We report here the enrichment and maintenance of a mixed culture derived from contaminated aquifer sediments that specifically degrades toluene and *o*-xylene under methanogenic conditions. The culture has been maintained with toluene or *o*-xylene as the sole sources of carbon and energy for more than 3 years. The growth and degradation kinetics and the effects of alternate electron acceptors and substrates are described and discussed in this paper. The results of a study on the metabolites of toluene degradation by this culture are presented in a separate paper (9).

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† Dedicated to the memory of Dunja Grbić-Galić (1950–1993).

MATERIALS AND METHODS

Aquifer material. Aquifer solids from Pensacola, Fla., were provided by E. M. Godsy (U.S. Geological Survey, Menlo Park, Calif.). The Pensacola aquifer consists of fine-to-coarse sand deposits, interrupted by discontinuous silts and clay. The upper 30 m of the aquifer is contaminated by creosote and pentachlorophenol. The samples were obtained from an actively methanogenic sandy zone of the aquifer, downgradient from the contamination source, from a depth of approximately 6 m. The groundwater at this depth contained tens of milligrams of nitrogen heterocycles, simple polyaromatic hydrocarbons, and phenols per liter. The indigenous microorganisms have been shown to anaerobically degrade aromatic and heterocyclic constituents of the water-soluble fraction of creosote (14, 15). Aseptic sampling was performed, and the aquifer core was stored (at 4°C) in sterile sealed containers previously flushed with argon (14).

Medium. A medium designed to support methanogenic bacteria that had the following constituents per liter of deionized water was prepared: 10 ml of phosphate buffer (27.2 g of KH_2PO_4 per liter, 34.8 g of K_2HPO_4 per liter), 10 ml of salt solution (53.5 g of NH_4Cl per liter, 7.0 g of $CaCl_2 \cdot 6H_2O$ per liter, 2.0 g of $FeCl_2 \cdot 4H_2O$ per liter), 2 ml of trace mineral solution [0.3 g of H_3BO_3 per liter, 0.1 g of $ZnCl_2$ per liter, 0.75 g of $NiCl_2 \cdot 6H_2O$ per liter, 1.0 g of $MnCl_2 \cdot 4H_2O$ per liter, 0.1 g of $CuCl_2 \cdot 2H_2O$ per liter, 1.5 g of $CoCl_2 \cdot 6H_2O$ per liter, 0.02 g of Na_2SeO_3 per liter, 0.1 g of $Al_2(SO_4)_3 \cdot 16H_2O$ per liter, 1 ml of H_2SO_4 per liter], 2 ml of $MgSO_4 \cdot 7H_2O$ solution (62.5 g/liter), 1 ml of redox indicator stock solution (1 g of resazurin per liter), 10 ml of saturated bicarbonate solution (260 g of $NaHCO_3$ per liter),

10 ml of filter-sterilized vitamin stock solution (0.02 g of biotin per liter, 0.02 g of folic acid per liter, 0.1 g of pyridoxine hydrochloride per liter, 0.05 g of riboflavin per liter, 0.05 g of thiamine per liter, 0.05 g of nicotinic acid per liter, 0.05 g of pantothenic acid per liter, 0.05 g of *p*-aminobenzoic acid per liter, 0.05 g of cyanocobalamin per liter, 0.05 g of thioctic acid per liter, 1 g of mercaptoethanesulfonic acid [coenzyme M] per liter), and 10 ml of an amorphous ferrous sulfide solution [39.2 g of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ per liter, 24.0 g of $Na_2S \cdot 9H_2O$ per liter] that had been washed three times with deionized water to remove free sulfide (4). The vitamins, bicarbonate, and ferrous sulfide were added from sterile stock solutions after the medium had been autoclaved and cooled while being gassed with N_2-CO_2 (80:20 [vol/vol]). The pH of the medium was usually between 6.8 and 7.0. In the experiments designed to test the effect of pH on degradation, the pH of the medium was adjusted to 6 with 1 N HCl or to 8 with 1 N NaOH.

Chemicals. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Aldrich Chemicals (Milwaukee, Wis.) and were greater than 99.9% pure. [*methyl*-^{14}C]toluene, [ring-^{14}C]toluene, and [*methyl*-^{14}C]*o*-xylene were also purchased from Sigma and had specific activities of 4.9, 9.5, and 10.9 mCi/mmol, respectively. The radiochemical purity of these compounds was about 99%.

Microcosms. Microcosms were prepared in 250-ml (8 oz.) screw-cap bottles and sealed with Mininert valves (Alltech Associates, Inc., Deerfield, Ill.). The bottles and caps were acid washed, sterilized, and brought into an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.). All glassware was preincubated in the glove box for at least 1 day to remove all traces of oxygen. Aquifer material (100 g) followed by 100 ml of medium was added to 250-ml bottles. Five groups of four microcosms were prepared. Each group consisted of a chemical control microcosm (medium plus substrates, no sediment), an autoclaved biological control microcosm (autoclaved sediment, medium plus substrates), and two replicate test microcosms. The sediment in sterile control bottles was autoclaved for 20 min at 121°C on 3 consecutive days before medium was added to these bottles. Each group was designed to test a different combination of substrates and concentrations. The first three groups were amended with a mixture of substituted monoaromatic compounds (toluene, *o*-xylene, *p*-xylene, and ethylbenzene) at either an initial aqueous concentration of 40 μM for each compound (first and second groups) or at an initial concentration of 320 μM for each compound (third group). The microcosms in the second group were additionally amended with 460 μM *p*-cresol. The fourth and fifth groups of microcosms were amended with the unsubstituted aromatic compounds, benzene and naphthalene (initial concentrations, 150 and 30 μM, respectively), with and without addition of 570 μM phenol. The putative intermediates, *p*-cresol and phenol, were added in some cases to attempt to stimulate growth of hydrocarbon-degrading organisms. All manipulations and incubations were performed in an anaerobic glove box (atmospheric composition: 85% N_2, 10% CO_2, and 5% H_2). The bottles were incubated statically at 35°C in the dark.

Enrichment cultures. After 8 months of refeeding active microcosms with toluene or *o*-xylene (approximately once per month with 1.5 to 3.0 μl of pure toluene or *o*-xylene), primary enrichment cultures were prepared by transferring sediment (10 g [wet weight]) and liquid (30 ml) from active microcosms to four clean, autoclaved 250-ml bottles. These bottles were then filled with 170 ml of prereduced, defined mineral medium and gassed with N_2-CO_2 (80:20 [vol/vol]). Two of these bottles were spiked with both toluene and *o*-xylene (as pure compounds) at 5 mg/liter (50 μM) each. The third was amended with toluene only (50 μM), and the fourth was amended with *o*-xylene only. Secondary enrichment cultures that no longer contained aquifer solids were prepared by inoculating fresh medium with some of the liquid from primary enrichment cultures (10 to 30% inoculum). Stable suspended cultures were maintained by refeeding weekly with toluene, *o*-xylene, or both (10 μl of toluene or *o*-xylene per 200 ml of culture) and by replacing 25 to 50% of the shaken culture medium with fresh medium approximately once every 4 months. Certain cultures were replenished with medium that had been filtered to remove the black precipitate, ferrous sulfide (FeS). After being replenished several times with filtered medium, the culture medium was devoid of solid precipitates that interferred with techniques such as protein measurement and microscopy.

Mass balance determinations. To determine the amount of methane formed from the degradation of toluene and *o*-xylene, a known amount of substrate was added to 200 ml of a freshly replenished culture flushed with a N_2-CO_2 gas mixture (to remove traces of H_2). The concentration of methane in the headspace was measured after the substrate was fully depleted. To determine the amount of CO_2 and nonvolatile products formed during the degradation of toluene and *o*-xylene, cultures were spiked with radiolabeled substrates ([^{14}C]toluene [both *methyl* and ring labeled] or [*methyl*-^{14}C]*o*-xylene), and the ^{14}C activities in the volatile, nonvolatile, and CO_2 fractions were determined as degradation proceeded, following the method described in reference 16. ^{14}C activity in liquid samples was determined with a Tricarb model 4530 scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.). Counting efficiency corrections were made with the external standard channels ratio method (1).

Kinetic experiments. The cell yield (Y) was determined in cultures growing in filtered medium by measuring the difference in the protein content of the culture before and after the degradation of a known amount of substrate. Protein content was determined by the method of Bradford (3) and converted to cell mass, assuming that 50% of the cell dry weight is protein. Substrate depletion was monitored for a series of initial substrate concentrations ranging from 50 μM to 2.5 mM. The initial active biomass concentration (X_0) was estimated from cell counts and protein determinations for samples of the culture taken at the start of the experiment. To determine cell counts, 10-μl samples were spread over a 1-cm^2 area on a microscope slide. The samples were heat fixed, stained with acridine orange (0.01%) for 2 min, and then washed with water. The cells were observed in oil immersion with an epifluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan) equipped with an Olympus FLPL ×100 objective lens and a ×10 ocular lens. Sixteen to twenty fields per sample were counted, and the average cell count per field was used to calculate the total cell count, given that the area of the field was 1.7×10^{-3} mm^2. The substrate depletion curves were fit to the Monod kinetic model (27) adapted for a volatile substrate in a two-phase system. The decay term (endogenous respiration) was neglected since decay rates are relatively small in anaerobic systems. Nonlinear regression of the data from substrate depletion curves to the Monod kinetic model provided estimates of the parameters K_s (half-saturation constant) and μ_{max} (maximum specific growth rate).

The Monod kinetic model for a volatile substrate is

$-dM/dt = (\mu_{max}/Y)\, X_0\, V_L\, [S/(K_s + S)]$, where M is the total mass of substrate in the incubation bottle at a given time (in milligrams), t is time (in days), μ_{max} is the maximum specific growth rate per day, Y is the yield (in milligrams per milligrams), X_0 is the initial active biomass concentration (in milligrams per liter), V_L is the liquid volume (in milliliters), S is the substrate aqueous concentration at a given time (in milligrams per liter), and K_s is the half saturation or affinity constant (in milligrams per liter).

The mass at any given time is related to the liquid concentration by the relationship $S = M/(V_L + H\, V_G)$, where H is the dimensionless Henry's Law constant for the substrate and V_G is the headspace volume (in milliliters).

Pasteurization. A culture (120 ml) that had been starved for 1 month and another culture (120 ml) that was actively degrading toluene were pasteurized at 80°C for 15 min. The cultures were cooled, dispensed into four 40-ml vials each (30-ml culture per vial), and refed toluene. Two of the four vials from each culture (starved and active) also received an inoculum of methanogens previously enriched with H_2 from the toluene-degrading culture (toluene was not degraded by these methanogens in control experiments). Toluene concentrations in pasteurized cultures were monitored over time in parallel with two sterile control vials to assess the survival of the members of the culture following the heat shock of pasteurization. The malachite green-staining procedure was used to determine whether endospores were present (7).

Cell freezing. Both toluene- and o-xylene-degrading stable cultures (200 ml of each) were centrifuged (6,000 × g, 45 min) in gas-tight bottles in a Damon/IEC (Needham, Mass.) model DPR-6000 centrifuge. Inside an anaerobic chamber, the supernatant was discarded, and the cell pellets were resuspended with 1 ml of filtered anaerobic medium in 1.5-ml Eppendorf tubes. Seventy microliters of dimethyl sulfoxide was added to each tube. The tubes were vortexed, removed from the glove box, and immediately flash frozen in liquid nitrogen. Frozen cells were stored at −80°C.

Electron microscopy. Toluene- and o-xylene-degrading enrichment cultures which had been transferred several times into filtered medium that no longer contained FeS precipitate were prepared for scanning electron microscopy. The cultures (100 ml) were centrifuged (6,000 × g, 45 min), and the pellets were resuspended in 1 ml of phosphate buffer (0.1 M, pH 7.2). The cultures were fixed with glutaraldehyde, stained with osmium tetroxide and uranyl acetate, and dehydrated in increasing strengths of ethanol (18). The samples were then dried with hexamethyldisilazane (HMDS; Polysciences, Inc., Warrington, Pa.) and examined with a Philips 505 scanning electron microscope.

Effects of alternate electron acceptors, alternate substrates, and inhibiting substances on toluene degradation. For each experiment, a 10 to 50% inoculum from a toluene-degrading enrichment culture was transferred to the required number of 40-ml vials. The volume was made up to 30 ml with fresh medium, and the vials were sealed with Mininert valves. In the experiments testing the effects of oxygen and nitrate, cultures growing in medium devoid of FeS precipitate were used. Oxygen was added by injecting air from a syringe into the vials, and the actual concentration added was calculated on the basis of the volume of air added, the Henry's Law constant and density of air, the percent oxygen in the air, and the liquid and gas volumes in the vial. Nitrate and sulfate were added as $NaNO_3$ and Na_2SO_4, respectively. Other substances tested as potentially stimulating alternate substrates included acetate, hydrogen, glucose, Casamino Acids (commercial mixture), sodium propionate, a fatty acid mixture (butyric, valeric, and caproic [both *n* and *iso*]), yeast extract, cysteine-HCl, and acetone. The toluene-degrading cultures were also challenged with cyclohexane, carbon tetrachloride, and benzene to assess the inhibitory effects of these compounds. Each of these test substances was added to duplicate cultures from neutralized aqueous stock solutions at different concentrations ranging over 2 orders of magnitude. Toluene or o-xylene was also added to these vials, at a fixed concentration of about 200 μM.

Analytical procedures. Toluene, xylenes, ethylbenzene, benzene, and cyclohexane concentrations were measured by withdrawing 300 μl of headspace from sample bottles with a 500-μl gas-tight syringe and injecting the headspace into a Fractovap 2900 series gas chromatograph (Carlo Erba Strumentazione, Milan, Italy) equipped with a photoionization detector (model PI-52-02; 10 eV lamp; HNU Systems, Inc.) and a DB-624 fused silica megabore capillary column (inside diameter, 30 by 0.53 mm; J&W Scientific, Folsom, Calif.). The operating conditions for the gas chromatograph-photoionization detector were an injection port temperature of 240°C, a detector temperature of 250°C, helium carrier gas at a column head pressure of 0.7 kg/cm^2, helium make-up gas at a flow of 30 ml/min, an isothermal temperature of 90°C, and a splitless injection (split closed for 30 s). The data from the gas chromatograph-photoionization detector were collected and processed with the Nelson Analytical, Inc., 3000 Series Chromatography Data System. The aqueous concentration of aromatic compounds in microcosms and enrichments was determined by comparing peak areas with those of standards. Standards for headspace analyses were prepared by spiking a methanolic stock solution of the aromatic compound into a Mininert-sealed bottle that contained 200 ml of water. The transfer of the stock solution was done with a gas-tight 500-ml syringe. The amount of stock solution added to the standard bottle was determined gravimetrically by weighing the syringe immediately before and after spiking. The aqueous concentration of aromatic compounds in standards was calculated by using Henry's Law constants obtained from reference 24. Methane concentrations were determined by injecting 400 μl of headspace onto a Fisher-Hamilton gas partitioner (model 25V; Fisher Scientific, Pittsburg, Pa.) equipped with a thermal conductivity detector and helium carrier gas (60 ml/min). Certified gas standards were used for calibration. p-Cresol, phenol, and naphthalene were analyzed by high-performance liquid chromatography (HPLC) (Series 400 Liquid Chromatograph; Perkin-Elmer Cetus, Norwalk, Conn.) equipped with a C_{18} reverse-phase column (inside diameter, 250 mm by 4.6 mm; Alltech Associates) and a 1050 Series Variable Wavelength Detector (Hewlett-Packard, Avondale, Pa.). Samples were centrifuged at 5,000 × g for 5 min to remove particulates before injection onto the HPLC. Data were collected and processed with a SP 4020 Data Interface (Spectra-Physics, Santa Clara, Calif.). The eluant was 70% acetate buffer (50 mM, pH 4.5) and 30% methanol at a flow rate of 1 ml/min (isocratic analysis); the detection wavelength was 280 nm.

RESULTS

Initial adaptation period. After long lag times, degradative activity was detected in some of the microcosms amended with substituted monoaromatic compounds at a low concentration (total concentration, 160 μM). p-Cresol degradation was complete in 80 days. Toluene and o-xylene transformation began in one of the two replicates from the group without p-cresol and in one of the two replicates from the

group supplemented with *p*-cresol. The lag times before the onset of degradation were 100 or 120 days for toluene and 200 or 255 days for *o*-xylene (with and without *p*-cresol, respectively). Upon refeeding these two microcosms with toluene and *o*-xylene simultaneously (100 μM each), degradation of both compounds began without a lag. In the microcosms amended with toluene, *o*-xylene, *m*-xylene, and ethylbenzene at a high concentration (total concentration, 1.3 mM), none of the compounds were transformed. In microcosms amended with benzene, naphthalene, and phenol, only phenol was degraded after 80 days of incubation. Neither benzene nor naphthalene was transformed after more than 300 days of incubation in microcosms with and without phenol. No significant depletion of aromatic compounds in the chemical control or autoclaved control microcosms was observed over the course of 300 days of incubation.

Enrichment cultures. Primary, secondary, and subsequent enrichment cultures retained the ability to degrade toluene and *o*-xylene to methane and carbon dioxide. In enrichment cultures that were always fed a mixture of toluene and *o*-xylene, both compounds were simultaneously degraded with no evidence of competition. However, cultures that were fed toluene only for more than 2 years could no longer degrade *o*-xylene, and vice-versa. The cultures readily degraded toluene and *o*-xylene but did not degrade *m*- or *p*-xylene. Over a period of 2 years, the rates of degradation of toluene or *o*-xylene increased from 5 μM/day to 50 μM/day, and the maximum substrate concentration degraded by these cultures also increased from about 200 μM to 2 mM. The optimum pH for degradation was found to be near 6.0 for both toluene and *o*-xylene, consistent with the fact that the pH of water in the Pensacola aquifer was 6 or less (15). At pH 7.0, the rate of toluene degradation was 75% of the rate at pH 6.0, and at pH 8.0 the rate of toluene degradation was 40% of the rate at pH 6. A decrease in temperature from 35 to 20°C caused a 25% reduction in the rate of toluene degradation.

Only methanogenic bacteria are known to produce methane. These bacteria exclusively metabolize simple one- or two-carbon compounds and hydrogen. Therefore, the complete methanogenic degradation of complex organic compounds such as toluene is believed to be carried out by the cooperative interaction of several groups of bacteria. Syntrophic relationships, such as interspecies H_2 transfer, enable reactions that would otherwise be thermodynamically unfavorable (28). We attempted to isolate pure cultures of toluene- or *o*-xylene-degrading organisms from the mixed cultures, using anaerobic roll tubes as described by Hungate (19). Colonies were obtained after 3 to 4 weeks of incubation. Some colonies were transferred to liquid medium, and slow degradation of toluene was observed. However, the slow degradation of toluene was accompanied by methane production, indicating that the colonies were not pure cultures but a mixture of fermentative and methanogenic bacteria. We did not succeed in separating the members of this consortium, perhaps because of the very tight syntrophic relationships that probably exist between the various organisms in the culture.

Theoretical stoichiometry. The overall theoretical stoichiometric and energetic equations for toluene and *o*-xylene biodegradation under methanogenic conditions were developed following the method described by McCarty (26), assuming an efficiency of energy transfer of 60% (Table 1). This method is based on the assumption that a correlation exists between the free energy of reaction and cell yield. On this premise, we calculated that, theoretically, 4.32 mol of methane and 2.32 mol of carbon dioxide would be generated from the degradation of 1 mol of toluene, or that 62, 33, and 5% of the carbon from toluene would be converted to methane, CO_2 (or HCO_3^-), and cell material, respectively. With these equations, theoretical cell yields of 11 g of cells (dry weight) per mol of toluene and 13 g of cells per mol of xylene were calculated. The amount of energy available for microorganisms from the oxidation of toluene or *o*-xylene under methanogenic conditions is considerably (about 17 times) less than that for toluene or xylene oxidation under aerobic conditions (Table 1).

TABLE 1. Stoichiometry and energetics of toluene and *o*-xylene oxidation under methanogenic conditions

Type of equation	Aromatic compound	Equation
Stoichiometry[a]	Toluene	$C_7H_8 + 7.102\ H_2O + 0.072\ NH_4^+ \rightarrow 2.318\ HCO_3^- + 4.32\ CH_4 + 2.39\ H^+ + 0.072\ C_5H_7O_2N$
	o-Xylene	$C_8H_{10} + 7.788\ H_2O + 0.084\ NH_4^+ \rightarrow 2.540\ HCO_3^- + 5.04\ CH_4 + 2.624\ H^+ + 0.084\ C_5H_7O_2N$
Energy[b]	Toluene	$C_7H_8 + 7.5\ H_2O \rightarrow 4.5\ CH_4 + 2.5\ HCO_3^- + 2.5\ H^+$
	o-Xylene	$C_8H_{10} + 8.25\ H_2O \rightarrow 5.25\ CH_4 + 2.75\ HCO_3^- + 2.75\ H^+$

[a] Includes cell ($C_5H_7O_2N$) formation.
[b] The data for computing free energy changes ($\Delta G^{o\prime}$) were taken from the studies by McCarty (26) and Thauer et al. (31). For toluene and *o*-xylene, $\Delta G^{o\prime}$ were −31.2 kcal/mol (ca. −131 kJ/mol) and −40.3 kcal/mol (ca. −169 kJ/mol), respectively. For comparison, the $\Delta G^{0\prime}$ values for the aerobic oxidation of toluene and xylene are −910 kcal/mol (ca. −3,810 kJ/mol) and −1,060 kcal/mol (ca. −4,435 kJ/mol), respectively.

Mass balances. Measured methane concentrations were consistently between 85 and 100% of the theoretical methane

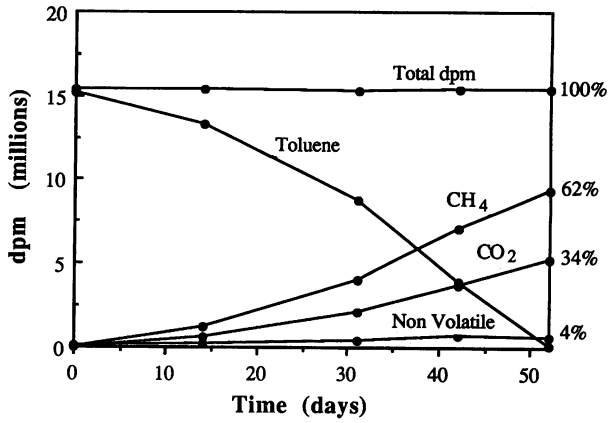

FIG. 1. ^{14}C label distribution in mixed methanogenic cultures fed [ring-^{14}C]toluene. ^{14}C in the volatile (toluene), nonvolatile (biomass and nonvolatile intermediates), and CO_2 fractions was measured directly. ^{14}C in methane was calculated, by using theoretical stoichiometry, from the mass of toluene degraded at each sampling time. dpm, disintegrations per minute.

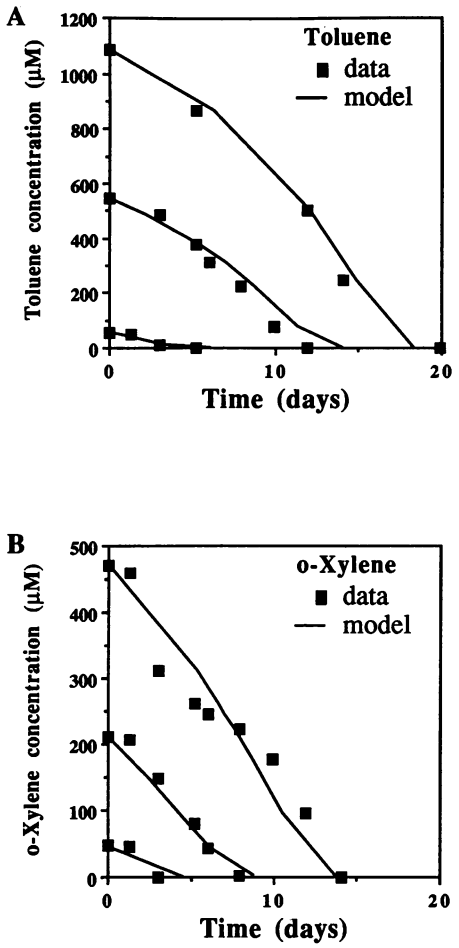

FIG. 2. Kinetics of toluene (A) and *o*-xylene (B) degradation. Experimental data were plotted with Monod kinetic model predictions. The biomass yield (Y) and the initial biomass concentration (X_0) were measured experimentally. The Monod parameters K_s (half-saturation constant) and μ_{max} (maximum specific growth rate) were approximated by nonlinear regression of the data from substrate depletion curves to the Monod model. The Monod model parameters for toluene were as follows: Y = 17 g of cells per mol of toluene, X_0 = 6.5 mg/liter, K_s = 30 μm, and μ_{max} = 0.11 day^{-1}. The Monod model parameters for *o*-xylene were as follows: Y = 17 g of cells per mol of *o*-xylene, X_0 = 8.4 mg/liter, K_s = 20 μm, and μ_{max} = 0.07 day^{-1}.

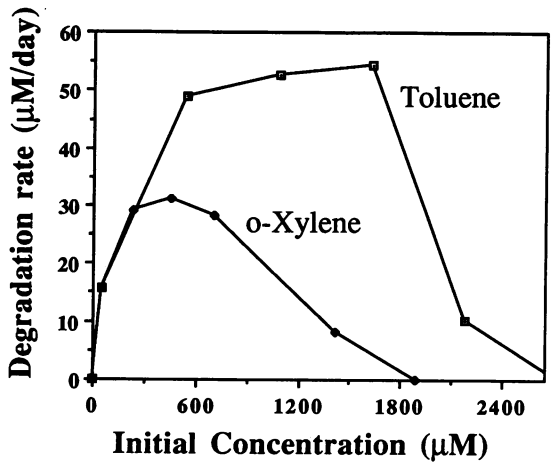

FIG. 3. Rate of degradation versus initial substrate concentration in mixed methanogenic cultures enriched with either toluene or *o*-xylene.

concentration (based on equations in Table 1) for both toluene and *o*-xylene in 22 different incubations over the three years of this study. Radiolabeled toluene and *o*-xylene were used to confirm the formation of CO_2 (or HCO_3^-). The ^{14}C label distribution shown in Fig. 1 was obtained from duplicate cultures fed [ring-^{14}C]toluene. The radioactivity in the volatile fraction (toluene), the nonvolatile fraction, and the CO_2 fraction was measured over time. For the volatile compounds CO_2 and toluene, the total mass in the bottle (aqueous phase and headspace) was the sum of the counts obtained in samples from the liquid phase and the corresponding counts in the headspace determined by using the Henry's Law constants for CO_2 and toluene. It was difficult to measure the radioactivity associated with methane because methane partitions almost completely into the headspace and is very poorly trapped in scintillation fluid. Since we found that near stoichiometric amounts of methane were produced in experiments with unlabeled substrates, we calculated the theoretical yield of radiolabeled methane from the measured amount of labeled toluene degraded at each time point and plotted these calculated [^{14}C]methane values in the same figure (Fig. 1). A near perfect mass balance was obtained upon summing up the radioactivity in the various fractions (measured and theoretical). Experiments with *methyl*-labeled toluene or *methyl*-labeled *o*-xylene also yielded near stoichiometric amounts of labeled CO_2.

Kinetics. Over a period of 2 years, the rate of degradation in enrichment cultures increased 10-fold predominantly as a result of the increased biomass concentration. From protein measurements, we estimated the cell yield (Y) to be about 17 g of cells per mol of toluene or *o*-xylene (standard deviation = 6.2, n = 8). The observed yield of 17 g/mol is marginally greater than the predicted theoretical yield of 11 to 13 g/mol and may indicate that the actual energy transfer efficiency is greater than 60% (assumed in theoretical calculations) and might be closer to 80%. Anaerobic systems have been shown to have higher energy transfer efficiencies (26). Substrate depletion curves for toluene and *o*-xylene were obtained for a range of initial substrate concentrations and for a given initial biomass concentration. The initial biomass concentration in these substrate depletion experiments was estimated from protein measurements and was assumed to be the active biomass concentration (X_0). We measured an initial biomass concentration of 6.5 mg/liter and 8.4 mg/liter for the toluene and *o*-xylene depletion experiments, respectively. The data from substrate depletion curves for toluene and *o*-xylene were fit to the Monod kinetic model (without a decay term) by nonlinear regression (Fig. 2) to derive values for the half-saturation constant (K_s) and the maximum specific growth rate (μ_{max}). This procedure yielded estimates for K_s of 30 and 20 μM (± 30%) and for μ_{max} of 0.11 day^{-1} and 0.07 day^{-1} (± 20%) for toluene and *o*-xylene, respectively. The doubling times for the stable consortia growing on toluene or *o*-xylene were therefore about 6 and 10 days, respectively. Typically, toluene was degraded more quickly than *o*-xylene, consistent with these kinetic parameters. Because growth was slow, the rate of degradation depended strongly on the initial biomass concentration. At initial substrate concentrations higher than those plotted in

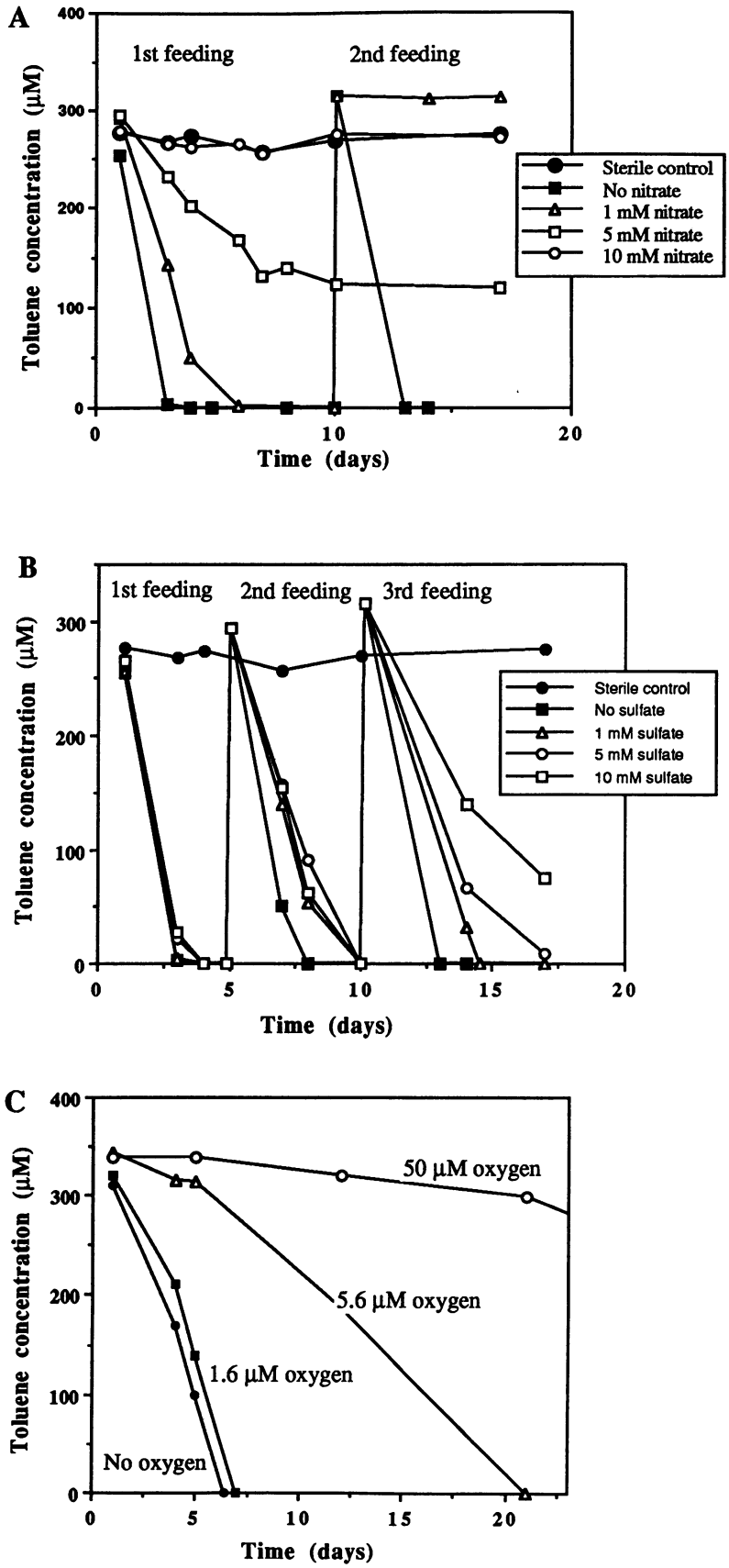

FIG. 4. Inhibition of toluene degradation by alternate electron acceptors. (A) Nitrate ($NaNO_3$); (B) sulfate (Na_2SO_4); (C) oxygen (O_2). Data are the averages of measured toluene concentration, in duplicate vials. Toluene and the alternate electron acceptor were added to the culture vials initially (first feeding). For the second and third feedings, only toluene was added to the vials.

Fig. 2, the data no longer fit the model because the rates of degradation were considerably lower as a result of substrate toxicity. Substrate toxicity was observed at an initial concentration of 700 μM for *o*-xylene, but not until the concentration was increased above 1,800 μM for toluene (Fig. 3).

Effect of alternate electron acceptors on toluene degradation. The addition of exogenous electron acceptors, such as nitrate or sulfate, slowed down or completely inhibited the degradation of toluene depending on their concentration (Fig. 4A and 4B). Nitrate was more inhibitory than sulfate at the same concentration. Oxygen was extremely toxic to this consortium (Fig. 4C), significantly inhibiting degradation at a concentration of 5.6 μM (0.18 mg/liter) and completely inhibiting degradation at a concentration of 50 μM (1.6 mg/liter).

Survival after starvation, pasteurization, or freezing. Cultures were able to survive for extended periods of time (at least 1 year) without any added toluene. Upon refeeding of the cultures, toluene degradation was initially very slow, but the activity was recovered and the rate of degradation eventually returned to prestarvation levels. Toluene-degrading activity was lost after pasteurization of active cultures. However, toluene-degrading activity was maintained after pasteurization of cultures that had been starved beforehand, with or without inoculation with methanogens after pasteurization, suggesting that all the members of the community degrading toluene (including the methanogens) were able to withstand mild heat shock, provided that the culture had been stressed beforehand to induce some form of sporulation or cell resistance. Microscopic observation confirmed the presence of some spore-forming microorganisms in stressed cultures. Cultures that were flash frozen, stored at −80°C, and later revived by thawing the frozen aliquot in sterile anaerobic medium retained the ability to degrade toluene and *o*-xylene.

Effect of alternate substrates on toluene degradation. To determine whether the presence of carbon sources other than toluene would stimulate or inhibit toluene degradation, a variety of test compounds in addition to toluene were fed to the consortium. Overall, none of the compounds tested stimulated degradation of toluene. Acetate, hydrogen, methanol, glucose, propionate, fatty acids, Casamino Acids, yeast extract, and cysteine-HCl were immediately used by the consortium as growth substrates preferentially over toluene. Only when the test compound was nearly completely degraded did the degradation of toluene begin. These inhibitory effects are illustrated for acetate (Fig. 5A) and glucose (Fig. 5B). Similar trends were observed for the other compounds tested, which were immediately used as substrates. Acetone had initially no effect on toluene degradation because acetone was being used only very slowly by the culture. However, after all the toluene and acetone from the first feeding had been consumed, the cultures were refed both acetone and toluene; and this time, acetone degradation began immediately (presumably because the cultures had adapted to this substrate) and toluene degradation was inhibited (Fig. 5C). Low concentrations of interspecies metabolites such as H_2 and acetate are believed to drive overall degradative reactions in methanogenic systems (28). The accessory organic compounds tested were degraded more rapidly than toluene and preferentially over toluene and may have caused transient build-up of acetate and H_2 to concentrations at which the degradation of toluene is no longer energetically favorable. In fact, acetate was observed to build up during the degradation of glucose and propionate to concentrations that would inhibit toluene metabolism as

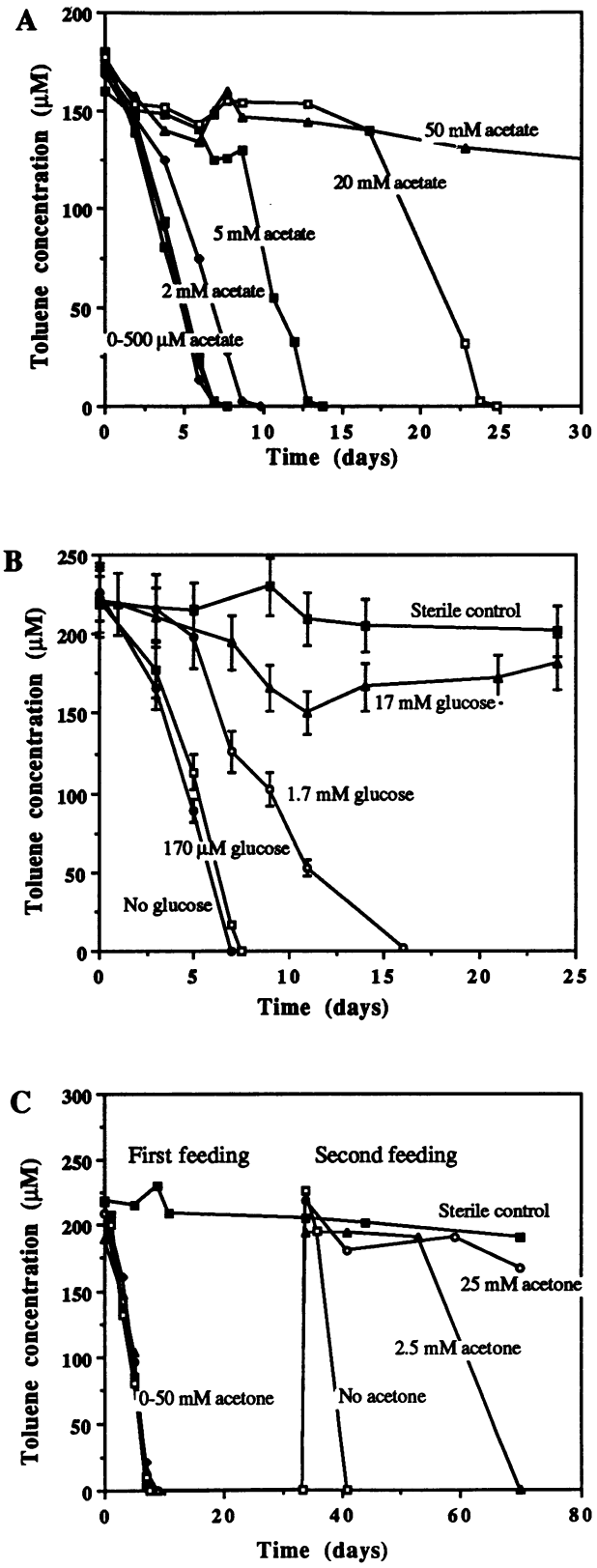

FIG. 5. Inhibition of toluene degradation by alternate electron donors. (A) Acetate; (B) glucose; (C) acetone. Error bars indicate standard deviations.

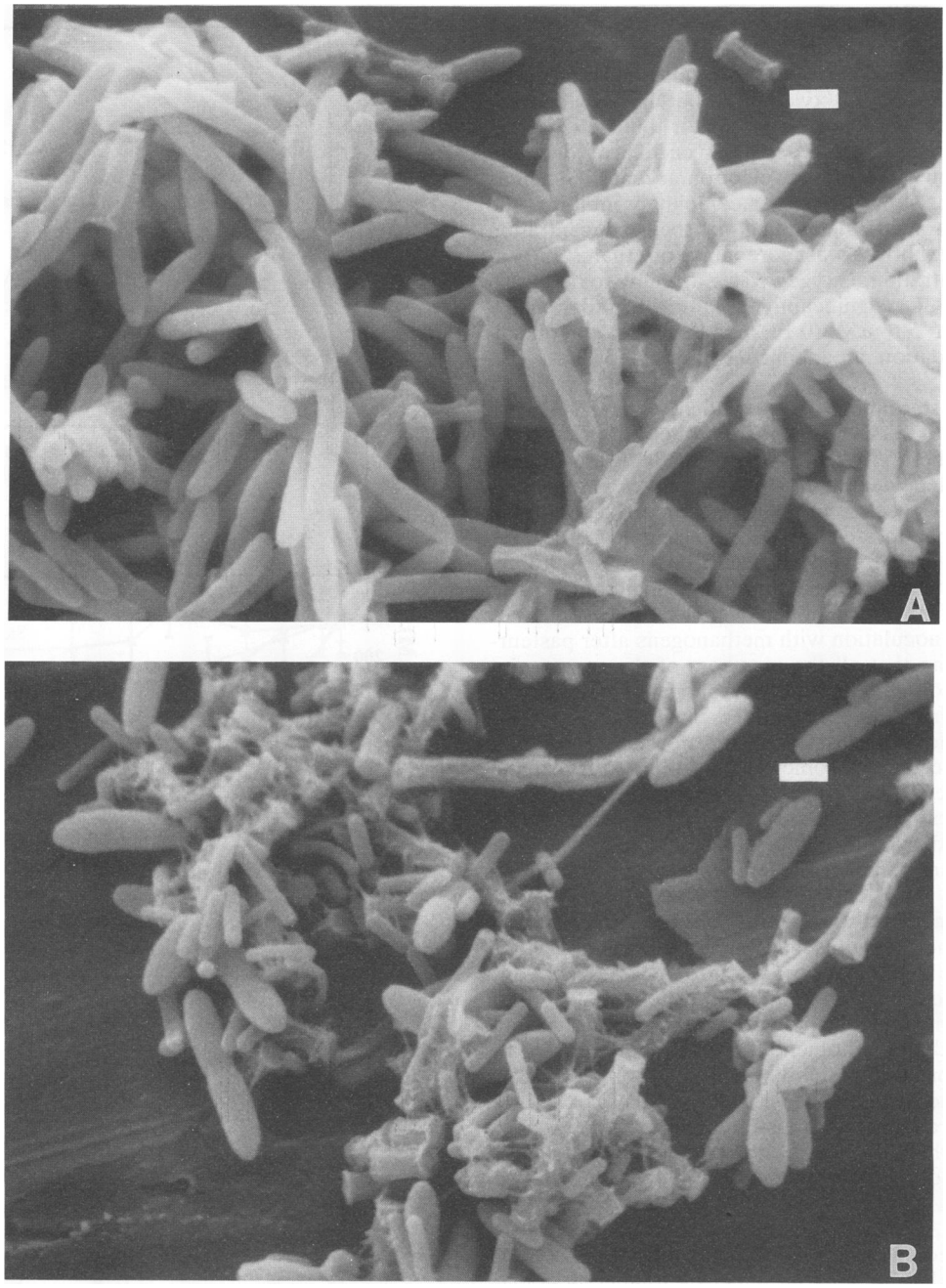

FIG. 6. Scanning electron micrographs of methanogenic cultures. (A) Toluene degrading; (B) *o*-xylene degrading. Bar = 1 μm.

shown in Fig. 5A. Acetate was also detected as a transient intermediate during toluene degradation (maximum concentration, 70 μM) and would accumulate in bromoethanesulfonate-inhibited cultures to concentrations up to 300 to 400 μM (bromoethanesulfonate is a specific inhibitor of methanogenesis [16]).

Some of the compounds tested were toxic at certain concentrations and were not metabolized. This was the case for cyclohexane, carbon tetrachloride, and benzene. Cyclohexane and carbon tetrachloride inhibited toluene degradation at concentrations above 700 and 40 μM, respectively. The toxic effect of some substances was dependent on the length of time exposed to the compound. This was demonstrated by amending cultures with benzene in addition to their normal substrates of toluene or *o*-xylene and observing the rate of degradation as a function of days exposed to benzene. The rate of toluene or *o*-xylene degradation was initially unaffected by the presence of benzene (0.7 to 3 mM). However, the rate of degradation gradually decreased to zero over a period of 50 to 100 days of incubation in the presence of benzene. The rate of degradation in parallel cultures not exposed to benzene increased over the same time period. The toxicity of the substrates toluene and *o*-xylene was also dependent on the length of time of exposure to these compounds. We observed that the maximum substrate concentration that could be degraded by the

culture depended on the initial cell density. At higher cell densities, higher initial toluene or *o*-xylene concentrations were tolerated, because the rate of depletion of the substrate was higher. Faster substrate depletion resulted in shorter exposure of the culture to high concentrations of substrates. At a low cell density and high substrate concentration, cell death from substrate toxicity was faster than cell growth.

Electron microscopy. Stable consortia growing on either toluene alone or *o*-xylene alone were observed by scanning electron microscopy. Rod-shaped cells predominated, confirming observations by light and epifluorescence microscopy. Two different types of rod-shaped cells (both with a diameter of 0.5 μm) were clearly visible in the culture degrading toluene (Fig. 6A): one morphology had rounded ends, while the other had blunt ends abutting the next cell in a chain. These two cell morphologies were also present in the *o*-xylene-degrading culture (Fig. 6B), although the round-ended rods appeared fatter and more ellipsoid than the rods of similar diameter in the toluene-degrading culture. A rod-shaped cell of narrower diameter (0.3 μm) was much more prevalent in the *o*-xylene-degrading culture than in the toluene-degrading culture. A notable difference between the micrographs of these two cultures was the presence of a large web of exopolysaccharide-like substance in the *o*-xylene-degrading culture. This web was absent in samples observed from the toluene-degrading culture. The blunt-ended, rod-shaped cell observed in both micrographs resembles the obligate acetate-utilizing methanogen, *Methanothrix soehngenii*, first isolated by Zehnder (12, 35).

DISCUSSION

Several findings from this study have important implications on the fate of monoaromatic compounds in anaerobic contaminated sites. Long adaptation periods may be required before the onset of detectable levels of biodegradation. Mutation or enzyme induction may have occurred during the adaptation period, but the most important factor contributing to the long adaptation period was most likely the very small number of active microorganisms in the sediment initially and the heterogeneity in the distribution of active microflora. The cultures enriched during this study appeared to be extremely substrate specific. Although monoaromatic compounds are structurally similar, only toluene and *o*-xylene were degraded; *m*-xylene, *p*-xylene, ethylbenzene, benzene, and naphthalene were not degraded. The addition of electron acceptors (such as nitrate or sulfate) to contaminated anaerobic sites will not necessarily accelerate degradation if the indigenous microbial communities are acclimated to the conditions of methanogenic fermentation. A very important factor influencing the anaerobic biotransformation of toluene and *o*-xylene was the presence of other organic compounds, either natural organic compounds or other components of pollutant mixtures. The addition of preferential microbial electron donors (such as acetone, methanol, glucose, fatty acids, and amino acids) inhibited toluene degradation in our experiments. These compounds occur naturally or are frequent cocontaminants, and their presence at a contaminated site may prevent the degradation of compounds that are more difficult to degrade, such as toluene and xylene. Many components of pollutant mixtures are toxic to microorganisms at certain concentrations. The degree of toxicity is dependent on the substance itself, the concentration, and on the length of time that the microorganisms are exposed to the toxic substance. Microorganisms have evolved defense mechanisms for surviving inhospitable conditions in the subsurface. The microorganisms enriched in this study were shown to withstand starvation and heat. These same defense mechanisms may offer these organisms some protection against many other forms of stresses present in natural and contaminated environments.

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